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(54) Title: CONTROL OF PLANT ABSCISSION AND POD DEHISCENCE			
(57) Abstract Recombinant or isolated nucleic acid sequences which: a) encode enzymes or other proteins involved in plant abscission or dehiscence; b) contain a promotor or other regulatory sequence which naturally controls expression of a gene involved in plant abscission or dehiscence; c) when introduced into a plant, prevent or otherwise interfere with normal plant abscission or dehiscence; or d) hybridise under stringent conditions to nucleic acid satisfying criterion (a), (b) or (c) or would do so but for the degeneracy of the genetic code are useful in regulating abscission and dehiscence, particularly in reducing pod shatter in <i>Brassica napus</i> .			

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CONTROL OF PLANT ABSCISSION AND POD DEHISCENCE

This invention relates generally to the control of plant abscission and pod dehiscence or shatter.

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Abscission is the process that causes the shedding of a range of plant parts, including leaves, flowers and fruit. The process occurs at precise sites and involves coordinated cell wall breakdown. Associated with cell separation is an increase in the activity of several hydrolytic enzymes including β -1,4-glucanase (cellulase, EC 3.1.2.4) and polygalacturonase (PG EC 3.2.1.15).

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The process of pod dehiscence, or shatter as it is commonly termed, in oilseed rape (*Brassica napus*) and other crops shares a number of features with abscission. Degradation and separation of cell walls occurs along a discrete layer of cells, termed the dehiscence zone, and a localised increase in the activity of cellulase has been reported prior to the onset of dehiscence (Meakin and Roberts *J. Exp. Bot.* 41(229) 995-1002 (1990) and *J. Exp. Bot.* 41(229) 1003-1011 (1990)). This process is agronomically important because it may result in the premature shedding of seed before the crop can be harvested. Adverse weather conditions can exacerbate the process resulting in a greater than 50% loss of seed. This loss of seed not only has a dramatic effect on yield but also results in the emergence of the crop as a weed in the subsequent growing season.

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Attempts to solve this problem over the last 20 years have focused on the breeding of shatter-resistant varieties. The most commonly used method is by trying to introduce germplasm from related species by interspecific hybridisation. Related species such as *B. nigra*, *B. juncea* and *B. campestris* have been used for this purpose but resulting plants from these crosses are frequently sterile and lose favourable

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characteristics which have to be regained by back crossing. This is both time consuming and laborious. The interspecific hybridisation strategy also has to cope with transferring two or more genes which are recessive in action into each of the breeding lines. Indeed, even within *B. campestris*, different genetic backgrounds have revealed different numbers of genes to be important in shatter resistance. This has necessitated breeders performing test crosses at each generation during the attempt to produce elite material. These difficulties have been compounded by the fact that shattering is a difficult and time-consuming trait to assess in the field. All these factors may account for the fact that the conventional breeding approach has made no progress over the last twenty years.

Other methods employed to try and alleviate the problem include chemicals, in the form of desiccants and pod sealants. The most widely used method to try and prevent seed loss is the mechanical technique of swathing in order to get uniform desiccation of the crop and reduce shattering by wind which occurs in the upright crop.

This invention takes a completely different approach to solve the problem of dehiscence and the related problem of adequately controlling plant abscission: it involves the use of recombinant DNA technology. In 1988, when plant biotechnology had reached an age of some considerable sophistication, Roberts and Taylor speculated:

By regulating cell separation at abscission sites, it may be possible...to also influence related processes such as pod dehiscence. (*Proceedings of the Symposium on the Physiology of Fruit Drop, Ripening, Storage and Post-Harvest Processing of Fruits*, Turin, 3-4 October 1988, pp 24-33).

However, without any indication of which genes may be involved in such processes, this exhortation did little to enable the art to address the problem at the cell or genetic level.

5 It has now been discovered that there are genes whose expression is, spatially and/or temporally, specific or at least preferential for tissue involved in abscission or dehiscence. The invention relates to the exploitation of such genes and related DNA sequences (including regulatory sequences) in the manipulation of plant abscission in general
10 and the reduction or prevention of pod dehiscence in particular.

According to a first aspect of the present invention, there is provided a recombinant or isolated nucleic acid sequence which:

- 15 (a) encodes an enzyme or other protein involved in plant abscission or dehiscence;
- (b) contains a promoter or other regulatory sequence which naturally controls expression of a gene involved in plant abscission or dehiscence;
- (c) when introduced into a plant, prevents or otherwise
20 interferes with normal plant abscission or dehiscence; or
- (d) hybridises under stringent conditions to nucleic acid satisfying criterion (a), (b) or (c) or would do so but for the degeneracy of the genetic code.

25 The recombinant or isolated nucleic acid will generally be DNA, but RNA is not excluded from the scope of the invention.

At its broadest, the invention is applicable generally to plant abscission or dehiscence, that is to say to the organised shedding of a part of a plant
30 by means of an absciss layer or dehiscence zone. Parts of plants that may from time to time be involved in abscission include leaves, petals, pods, seeds and fruit. The invention may also have application in

regulating the abscission of pollen from anthers, which may be useful in generating artificially male sterile plants, which are useful for hybrid seed production (as, for example, discussed in WO-A-9211379).

5 In the embodiments of the invention relating to dehiscence, the invention has application to all crops that lose seed pre-harvest because of cell separation events. An economically important crop to which the invention applies is *Brassica napus*.

10 Recombinant or isolated nucleic acid sequences which satisfy criterion (a) given above are illustrated by the nucleic acid sequence of Figures 3 and 8, which encode the amino acid sequences shown in those figures. All other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequences are also
15 preferred embodiments of the invention. Nucleic acid sequences which are substantially homologous to nucleic acid sequences encoding the amino acid sequences shown in Figures 3 and 8 also constitute preferred embodiments of the invention. "Substantial homology" may be assessed either at the nucleic acid level or at the amino acid level. At the nucleic
20 acid level, sequences having substantial homology may be regarded as those which hybridise to the nucleic acid sequences shown in Figures 3 and 8 under stringent conditions (for example at 35 to 65°C in a salt solution of about 0.9M). At the amino acid level, a protein sequence may be regarded as substantially homologous to another protein sequence if
25 a significant number of the constituent amino acids exhibit homology. At least 40%, 50%, 60%, 70%, 80%, 90%, 95% or even 99%, in increasing order of preference, of the amino acids may be homologous. Specifically excluded from nucleic acids satisfying the criterion of (a) are nucleic acids encoding the DC2.15 and pZRP3 sequences shown in Figure
30 5. Nucleic acids encoding plant abscission or dehiscence proteins having one or more amino acids of pSAC51 not shared with at least one of DC2.15 and pZRP3 are, however, within the scope of the invention.

The most preferred embodiments of the invention satisfying criterion (a) are nucleic acids encoding proteins specifically or at least preferentially expressed in the dehiscence zone of pericarp tissue, particularly in *Brassica napus*.

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Nucleic acid satisfying criterion (b) given above constitutes a powerful and flexible means of achieving the benefits of the invention. Such nucleic acid contains a promoter or other regulatory sequence which naturally controls expression of a gene involved in plant abscission or dehiscence. The promoter is that region of a gene which regulates its expression, for example by specifying the time or location of expression. Promoters can be separated from the coding region of a gene and used to drive a different coding region, thus allowing the expression of a different product.

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In one implementation of the invention under this general heading, the promoter may drive a gene which disrupts cellular development. For example, the promoter may drive DNA coding a lytic enzyme. The lytic enzyme may cause lysis of one or more biologically important molecules, such as macromolecules including nucleic acid, protein (or glycoprotein), carbohydrate and in some circumstances lipid.

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Ribonuclease (such as RNase T1) and barnase are examples of enzymes which cause lysis of RNA. Examples of enzymes which lyse DNA include exonucleases and endonucleases, whether site specific (such as *EcoRI*) or non-site-specific. Glucanase is an example of an enzyme which causes lysis of a carbohydrate. Lipases whose corresponding nucleic acids may be useful in the invention include phospholipase A₂. Actinidin is an example of a protease, DNA coding for which may be useful in the invention; other examples include papain zymogen and papain active protein. Such "killer" enzymes as these do not have to be lytic enzymes. Other examples of enzymes DNA coding for which may be useful in the

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invention catalyse the synthesis of phytohormones, such as isopentenyl transferase, which is involved in cytokinin synthesis, and one or more of the enzymes involved in the synthesis of auxin.

5 While promoters useful in feature (b) of the invention may drive DNA encoding an enzyme, they may alternatively drive DNA whose transcription product is itself deleterious. Examples of such transcription products include antisense RNA and ribozymes.

10 As far as antisense nucleic acid is concerned, introducing the coding region of a gene in the reverse orientation to that found in nature can result in the down-regulation of the gene and hence the production of less or none of the gene product. The RNA transcribed from antisense DNA is capable of binding to, and destroying the function of, a sense RNA of
15 the sequence normally found in the cell, thereby disrupting function. Examples of such antisense DNAs are the antisense DNAs of the sequences shown in Figures 3 and 8. Since these genes are normally expressed in the dehiscence zone, antisense to them may be expected to disrupt normal dehiscence.

20 Ribozymes are RNA "enzymes" capable of highly specific cleavage against a given target sequence (Haseloff and Gerlach, *Nature* 334 585-591 (1988)).

25 Promoters useful in feature (b) of the invention may be located in cDNA or genomic libraries using, for example, probe sequences taken from the nucleic acid sequences of Figures 3 and 8.

30 A third category of nucleic acid useful in the invention is identified under (c) above, as that which, when introduced into a plant, prevents or otherwise interferes with normal plant abscission of dehiscence. Generally this would be achieved by interfering with the proper expression

of one or more genes involved in abscission or dehiscence. Of course, dehiscence- or abscission-specific promoters, as discussed under (b) above, may be useful in this feature of the invention. However, there is a broader dimension which must be considered: antisense DNA or ribozyme-encoding DNA specific for abscission- or dehiscence-specific genes need not be driven by abscission- or dehiscence-specific promoters. Instead, they could be driven by constitutive or other promoters (such as for example the CaMV 35S, rubisco or plastocyanin promoter). As the sense gene is only expressed in the pod, there will with an antisense approach be no pleiotropic effects on plant development, and only the development of the dehiscence zone will be disruptive. A ribozyme gene expressed throughout the plant will not result in a translated protein product, and so may require less metabolic energy than the synthesis of a gene product throughout most of the plant.

Antisense technology and ribozyme technologies have already found application in other areas of plant molecular biology. For example, antisense technology has been used to control tomato fruit ripening. Ribozyme technology has been used to control viral infection of melons.

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While DNA or RNA in accordance with this feature of the invention generally interferes with the proper expression of a gene or genes, in preferred embodiments expression is substantially prevented.

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Another important group of nucleic acids useful in the invention is specified under feature (d); this includes nucleic acids which hybridise under stringent conditions to nucleic acids satisfying the criterion of one or more of features (a), (b) or (c). For example, nucleic acid fragments are useful for probing for similar genes involved in abscission or dehiscence. For example, an *Arabidopsis* or other gene library may be probed. Fragments of at least 10, 20, 30, 40 or 50 more nucleotides

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may be used. Many useful probes are from 15 to 20 nucleotides in length.

5 In preferred embodiments of DNA sequences of this invention, 3'-transcription regulation signals, including a polyadenylation signal, may be provided. Preferred 3'-transcription regulation signals may be derived from the cauliflower mosaic virus 35S gene. It should be recognised that other 3'-transcription regulation signals could also be used.

10 Recombinant DNA in accordance with the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and, preferably, to enable
15 selection of cells harbouring vectors incorporating heterologous DNA. Appropriate start and stop signals will generally be present. Additionally, if the vector is intended for expression, sufficient regulatory sequences to drive expression will be present; however, DNA in accordance with the invention will generally be expressed in plant cells, and so microbial host
20 expression would not be among the primary objectives of the invention, although it is not ruled out. Vectors not including regulatory sequences are useful as cloning vectors.

25 Cloning vectors can be introduced into *E. coli* or another suitable host which facilitate their manipulation. According to another aspect of the invention, there is therefore provided a host cell transfected or transformed with DNA as described above.

30 DNA in accordance with the invention can be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but recombinant DNA technology forms the method of choice.

Ultimately, DNA in accordance with the invention will where appropriate be introduced into plant cells, by any suitable means. According to a further aspect of the invention, there is provided a plant cell including DNA in accordance with the invention as described above.

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Preferably, DNA is transformed into plant cells using a disarmed Ti-plasmid vector and carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Alternatively, the foreign DNA could be introduced directly into plant cells using an electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledonous. Any other method that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would also be suitable. This includes species of plant which are not currently capable of genetic transformation.

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Preferably DNA in accordance with the invention also contains a second chimeric gene (a "marker" gene) that enables a transformed plant containing the foreign DNA to be easily distinguished from other plants that do not contain the foreign DNA. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella *et al.*, *EMBO J.* 2(6) 987-95 (1983) and Herrera-Estrella *et al.*, *Nature* 303 209-13 (1983)), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells other than the tapetum, thus allowing selection of cells or tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. However any other suitable second promoter could be used.

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5 A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides transgenic plants (or parts of them, such as propagating material) including DNA in accordance with the invention as described above. The regeneration can proceed by known methods.

10 A further aspect of the invention is constituted by novel proteins which are preferentially or exclusively expressed in dehiscence zones or abscission layers. Examples of such proteins are those whose amino acid sequences are given in Figures 3 and 8.

Preferred features of each aspect of the invention are as for each other aspect *mutatis mutandis*.

15 The invention will now be illustrated by the following Examples. The Examples refer to the accompanying drawings, in which:

20 FIGURE 1 relates to Example 1 and is a diagrammatic representation of a transverse section through an oilseed rape pod showing the distinction between 'zone' and 'non-zone' pericarp tissue used for protein extraction.

25 FIGURE 2 also relates to Example 1 and is a Northern blot analysis of total RNA (10 μ g). The RNA was hybridised to radiolabelled pSAC51 cDNA insert.

- a) 20 DAA pod samples given a 48 hour exposure time to illustrate signal in the non-zone sample.
- b) Later stages of pod development given 15 hour exposure.

30 Z = Zone, N = Non-Zone, DAA = days after anth sis, L = Leaf, R = Root, S = Seed.

FIGURE 3 also relates to Example 1 and shows the nucleotide and deduced amino acid sequence of pSAC51 cDNA. The initiation and termination codons are indicated by a single asterisk. Amino acid domains of interest are double underlined and a possible glycosylation site is underlined. A putative polyadenylation signal has asterisks above it.

FIGURE 4 also relates to Example 1 and shows the hydropathy profile of the cDNA clone pSAC51 deduced amino acid sequence. The profile was computer generated according to Kyte and Doolittle *J. Mol. Biol.* 157 105-132 (1982).

FIGURE 5 also relates to Example 1 and shows the sequence alignment of amino acids deduced from nucleotide sequences of the following cDNAs:

pSAC51 - Oilseed rape pods
DC2.15 - Carrot embryos, Aleith and
Richter *Planta* 183, 17-24 (1990)
pZRP3 - Maize roots, John *et al*, *Plant*
Mol. Biol. 20 821-831 (1992)

Common amino acids are in bold.

FIGURE 6 also relates to Example 1 and shows a genomic Southern blot analysis of *B. napus* DNA probed with the pSAC51 cDNA. 10µg DNA was digested using the following restriction enzymes: E = *EcoRI*, H = *HindIII*, B = *BamHI*. the positions of *HindIII*-digested λ DNA markers are also indicated.

FIGURE 7 refers to Example 2 and shows a Northern blot analysis of total RNA (10µg). The RNA was hybridised to radiolabelled pSAC25 cDNA insert. Z = Zone, N = Non-Zone, DAA = days after anthesis, L = Leaf, R = Root, S = Seed.

FIGURE 8 also refers to Example 2 and shows the pSAC40 nucleotide and deduced amino acid sequences.

5 FIGURE 9 also refers to Example 2 and shows a hydropathy profile of the cDNA clone pSAC25 deduced amino acid sequence. The profile was computer generated according to Kyte and Doolittle, *J. Mol. Biol.* 157 105-132 (1982).

10 FIGURE 10 also refers to Example 2 and shows a genomic Southern blot analysis of *B. napus* DNA probed with the pSAC25 cDNA. 10 μ g DNA was digested using the following restriction enzymes: E = *EcoRI*, H = *HindIII*, B = *BamHI*. the position of *HindIII* digested λ DNA markers are also indicated.

15 FIGURE 11A shows a restriction map of the *A. thaliana* genomic clone gSAC25. The position of the SAC25 coding region is shown as a filled box and the extent of the insert in pDH30 is indicated.

20 FIGURE 11B shows a restriction map of the *A. thaliana* genomic clone gSAC51. The position of the SAC51 coding region is shown as a filled box and the extent of the insert in pDH31 is indicated.

25 FIGURE 12 shows the construction of a chimeric gene that expresses SAC25 anti-sense RNA from the CaMV double 35S promoter in transgenic plants.

EXAMPLE 1 - Cloning of pSAC51

Plant Material

30 Seeds of *B. napus* cv Rafal were grown as described by Meakin and Roberts, (*J. Exp. Bot.* 41(229) 995-1002 (1990)) with the following modifications. Single seedlings were potted into 10cm pots, and after

vernalization, were re-potted into 21cm pots. At anthesis tags were applied daily to record flower opening. This procedure facilitated accurate age determination of each pod. Pods were harvested at various days after anthesis (DAA). The dehiscence zone (see Figure 1) was excised from the non-zone material and seed using a scalpel blade (Meakin and Roberts *J. Exp. Bot.* 41(229) 1003-1011 (1990)) and immediately frozen in liquid N₂ and stored at -70°C.

RNA Isolation

All chemicals were molecular biology grade and bought from either Sigma Chemical Ltd (Dorset, UK), or Fisons (Loughborough, UK). Total RNA was extracted using the polysomal extraction method of Christoffersen and Laties, *Proc. Natl. Acad. Sci.* 79 4060-4063 (1982), with the following alterations. The plant material was ground to a powder in liquid N₂ and then in 10 volumes of extraction buffer (200mM Tris-acetate [pH 8.2], 200mM magnesium acetate, 20mM potassium acetate, 20mM EDTA, 5% w/v sucrose, after sterilisation 2-mercaptoethanol was added to 15mM and cycloheximide added to a final concentration of 0.1 mg ml⁻¹). The supernatant was then layered over 8 ml 1M sucrose made with extraction buffer and centrifuged in a KONTRON[™] (Switzerland) TFT 70.38 rotor at 45,000rpm (150,000g) for 2 hr at 2°C in a Kontron CENTRIKON[™] T-1065 ultra-centrifuge. Pellets were then resuspended in 500µl 0.1M sodium acetate, 0.1% SDS, pH 6.0 and phenol/chloroform (1:1 v/v) extracted and the total RNA precipitated. Poly(A)⁺ RNA was isolated from total RNA extracted, from both the zone and non-zone tissue of 40, 45 and 50 DAA pods, using a Poly(A) Quik[™] mRNA purification kit (Stratagene, Cambridge, UK) following the manufacturers instructions, and then bulked together. Total RNA was also extracted from leaves, seeds and pods using a method described by Dean *et al*, *EMBO J.* 4 3055-3061 (1985) for use in Northern analyses.

cDNA Library Construction and Screening

A cDNA library was constructed using 5 μ g poly(A)⁺ RNA extracted from the dehiscence zone of pods prior to and during dehiscence. The library was constructed using the λ ZAP-cDNA synthesis kit according to the manufacturers' instructions (Stratagene). This resulted in the production of a library containing 1.2 X 10⁶ recombinants. Several plaques were picked at random and *in vivo* excised (Short *et al*, *Nucl. Acids Res.* 16 7583-7600 (1988)). The average insert size was 1 Kb. Differential screening was performed using single-stranded cDNA probes synthesised from poly(A)⁺ RNA isolated from dehiscence zone or non-zone pod material. The probes were synthesised using the method of Picton *et al*, *Plant Mol. Biol* in press (1993) and used to screen 40,000 recombinant plaques by *in situ* plaque hybridisation. Duplicate plaque lifts were obtained using HYBOND[™] N⁺ membranes (Amersham, Aylesbury, UK) and were then treated and hybridised according to manufacturers instructions but were washed at 65°C in 0.1 X sodium chloride, sodium phosphate, EDTA (SSPE), 0.1% SDS. Any plaques hybridising preferentially to zone probes were re-screened at densities of 50-100 plaques/plate. Chosen plaques were cored out of the plate and plasmids isolated using the *in vivo* excision procedure (Short *et al*, *Nucl. Acids Res.* 16 7583-7600 (1988)). Inserts were amplified by polymerase chain reaction (PCR) using the T3, T7 bacteriophage promoters and subsequently used for probes. Isolated plasmid was also used as a template for sequencing.

Northern Blot Analysis of RNA

10 μ g total RNA isolated from various parts of the oilseed rape plant were separated on a 1 X TBE, 1% agarose/6% formaldehyde denaturing gel. The RNA was transferred onto a nylon membrane (GeneScreen, NEN-Du Pont, UK) using capillary transfer. The gel, RNA samples, blot and hybridisation were performed in accordance with the membrane manufacturers instructions. A radio-labelled probe was generated using 100ng of insert from the plasmid pSAC51, using [³²P] dCTP (110 TBq

nmole⁻¹, Amersham) and a nick translation kit (Boehringer Mannheim, Lewes, UK). Unincorporated label was removed from the probe by passing it through a SEPHADEX™ G-50 column and eluting the probe with TE (pH 8). The blot was washed at 65°C in 0.1 X SSPE, 0.1% SDS and exposed to KODAK™ X-AR5 film with intensifying screens at -70°C.

Genomic DNA Isolation and Characterisation

DNA was isolated, using a miniprep procedure using a modified form of the extraction buffer described by Clarke *et al*, *Genome* 32 334-339 (1989). Young expanding oilseed rape seedlings were homogenised in a 3.8:0.6:0.6 mixture of the following; TNE buffer (0.05M Tris.HCl pH 7.5, 0.2M EDTA: 0.1M NaCl): 5% SDS: 1 mg ml⁻¹ proteinase K; to this solution was added sodium diethyldithiocarbamate and sodium bisulphite to 0.4% (w/v) just before use. The samples were then incubated for 1 hr at 37°C and debris removed by centrifugation in a microfuge at 11,600g for 5 min. The eluate was then extracted with equal volumes of phenol/chloroform (1:1 v/v) and then chloroform alone. Nucleic acids were then precipitated by the addition of 2.5 vols 95% ethanol containing 5% (v/v) 2M Na acetate, pH 5.5. The sample was then mixed and immediately centrifuged at 11,600g for 5 min. The resulting pellet was resuspended in 300µl TE, 10µl of RNaseA (10 mg ml⁻¹) added, and then incubated at 37°C for 15 mins before 300µl CTAB buffer (0.2M Tris.HCl pH 7.5, 0.05M EDTA, 2M NaCl and 2% w/v CTAB) was added before a further incubation at 60°C for 15 mins. Following re-extraction with an equal volume of chloroform the DNA was precipitated with an equal volume of isopropanol at -20°C. Subsequent digestions by restriction endonucleases were carried out as detailed in Stacey and Isaac, Restriction enzyme digestion, gel electrophoresis and vacuum blotting of DNA to nylon membranes (1993). The DNA was then separated in 1 X TBE, 0.8% agarose and transferred to GENESCREEN+™ (NEN) nylon membrane. The probes were made as described for Northern analysis and hybridisation was carried out according to manufacturers

recommendations. The final wash of the membrane was at 65°C in 0.1X sodium chloride, sodium citrate (SSC) 0.1% SDS.

DNA Sequencing

5 Plasmid DNA was isolated by the alkaline-lysis method (Sambrook *et al*,
Molecular Cloning: A Laboratory Manual New York, Cold Spring Harbour
Laboratory Press (1989)). Supercoiled plasmid DNA was isolated as
reported in the TAQTRACK™ sequencing manual (Promega Ltd,
Southampton, UK). 5µg denatured plasmid was sequenced using the
10 chain-termination method of Sanger *et al*, *Proc. Natl. Acad. Sci.* 74 5463-
5467 (1977) using the sequencing kit SEQUENASE™ v.2.0, (USB, c/o
Cambridge BioScience, UK). Compressions were resolved by performing
the reactions at 70°C using *Taq* DNA polymerase (TAQTRACK™ sequencing
kit, Promega). DNA sequences were analysed using the University of
15 Wisconsin Genetics Computer Group (UWGCG) package (Devereux *et al*,
Nucl. Acids Res. 12 387-395 (1984)) and the DNA Strider program
(Marck, *Nucl. Acids Res.* 16 1829-1836 (1988)).

Isolation of cDNA clones by differential screening

20 cDNA clones of mRNAs accumulating preferentially in the dehiscence
zones of developing pods were identified using a differential screening
strategy. This employed random-primed, radio-labelled cDNAs generated
from poly(A)⁺ RNA, isolated from the bulked 40, 45 and 50 DAA
samples of the pod containing the dehiscence zone and from adjacent
25 tissue lacking this zone (see Figure 1). Any potential positives were
confirmed by isolation and rescreening at lower densities. By this method
36 clones were isolated from screening 150,000 recombinants, which on
cross-hybridization could be grouped into 13 families. When screened
with the insert from the clone designated pSAC51, 19 other clones were
30 shown to have homology (data not shown), indicating that this cDNA
may encode an abundant mRNA. The insert from pSAC51 was

approximately 700 bp in length by comparison with DNA standards on an ethidium bromide stained agarose gel.

pSAC51 mRNA expression by Northern analysis

5 Pods were harvested at 20, 40, 45, 50, 60 DAA and dehiscence zone (Z) and flanking non-zone (NZ) tissue isolated (see Figure 1). Total RNA was extracted from these excised parts and from the seed, leaves and roots. Northern analysis revealed that the 700 bp insert from pSAC51 hybridised to a mRNA of about 700 nucleotides (See Figure 2). At 20 DAA
10 hybridisation was apparent in both Z and NZ, but subsequently disappeared in NZ tissues and preferentially accumulated in the dehiscence zone tissue with maximum signal occurring at 60 DAA. The process of dehiscence is visible to the naked eye at 50 DAA. The transcript could not be detected in the leaves, seeds and roots. The
15 cDNA pSAC51 was deemed to be near full-length because the mRNA transcript size was similar to that of the cDNA insert size.

pSAC51 sequence and amino acid analysis

Both strands of the cDNA were sequenced and the result is shown in
20 Figure 3. When sequenced the cDNA was 756 bp in length. The largest open reading frame (ORF) was 441 nucleotides in length beginning at position 15 (A) and ending at position 456 (T). The deduced protein sequence of 147 amino acids has a calculated molecular mass of approximately 15 kDa and rich in proline (14.2%), leucine (14.2%) and
25 lysine (10.2%). At the end of the nucleotide sequence there was a large poly(A) tail that encompassed 42 (A) nucleotides. A prominent feature of the pSAC51 deduced amino acid sequence was the arrangement of the proline residues into the repeated motif "Pro-X" (underlined in Figure 3). The sequence also contains a potential glycosylation site denoted by the
30 motif "N-X-S or T" (Kornfeld and Kornfeld, *Ann. Rev. Biochem.* 54 631-664 (1985)) and a possible polyadenylation site (Joshi, *Nucl. Acids Res.* 15 9627-9640 (1987)).

The hydropathy plot (Figure 4) of the peptide indicates that the protein has several distinct domains. The protein has a hydrophobic amino terminus, characteristic of a membrane spanning cleavable signal sequence (von Heijne, *Nucl. Acids Res.* 14 4683-4690 (1986)), extending to position 30. It then has a hydrophilic region extending to position 70, followed by a further hydrophobic region extending to the carboxy terminus.

Analysis of this sequence utilising the UWGCG programs revealed the protein to share significant homology with several proteins; 57% with a carrot cDNA (DC2.15) representing a mRNA that accumulates during somatic embryogenesis (Aleith and Richter, *Planta* 183 17-24 (1990)); 40% with a cDNA (pZRP3) that represents a mRNA localised to cortical cells in maize roots (John *et al*, *Plant Mol. Biol.* 20 821-831 (1992)). A comparison of the sequences of these proteins with that deduced from the nucleotide sequence of pSAC51 is shown in Figure 5. The deduced amino acids share similar compositions and hydropathy plots. They also have the "Pro-X" domain within the first 50 amino acids and a characteristic DALK motif underlined in Figure 5.

Genomic Southern analysis of pSAC51

The 756 bp insert of pSAC51 was used as a probe for hybridisation to Southern blots of *B. napus* genomic DNA digested with *EcoRI*, *HindIII* and *BamHI* (Figure 6). The probe hybridised to several fragments ranging in size from 5 kb to 1 kb. The cDNA has an internal restriction site for *HindIII* at nucleotide 32 and this may account for extra fragments.

Discussion

Pod dehiscence is a hard phenotype to measure accurately and therefore the precise start of the process is not known. Cellulase activity increases in the dehiscence zone from 40 DAA and precedes the first visible signs

of cell wall breakdown by 15-20 days (Meakin and Roberts, *J. Exp. Bot.* 41 1003-1011 (1990)). Therefore mRNA extracted from different developmental stages (40, 45 and 50 DAA) were bulked in order to increase the chances of obtaining mRNAs that are present prior to and during the process of dehiscence. As the pods develop on an oilseed rape plant they undergo growth and expansion (Meakin, The physiology of bud abscission and pod shatter in oilseed rape (*Brassica napus* L.) PhD Thesis, University of Nottingham, UK (1988)) which then stops at 20 DAA. After this point the pods do not increase in size as the priority becomes seed expansion and filling. During this time the pods are drying out and losing chlorophyll (Meakin, The physiology of bud abscission and pod shatter in oilseed rape (*Brassica napus* L.) PhD Thesis, University of Nottingham, UK (1988)). Concurrent with this is the process of pod dehiscence (Meakin and Roberts, *J. Exp. Bot.* 41 995-1002 (1990)).

This example relates to the isolation of a cDNA clone that corresponds to a mRNA that preferentially accumulates in the dehiscence zone of the developing pod (Figure 2). The pSAC51 mRNA is present at the early stages of pod development in both the zone and non-zone tissue. The presence of signal in the non-zone RNA at 20 DAA cannot be fully explained. Genomic Southern analysis (Figure 6) shows that pSAC51 may be controlled by a single or a small family of genes and the presence and/or arrangement of introns has yet to be determined. therefore the presence of some hybridising bands may be due to further restriction sites in the genomic sequence. also *B. napus* (n=19) is an amphidiploid resulting from a natural interspecific hybridisation even between *B. oleracea* (n=9) and *B. campestris* (n=10) and the resulting genome is likely to contain 2 copies of any gene, one from each parental genome.

The deduced protein sequence of pSAC51 has several features that are worth mentioning (Figure 3). the protein is rich in the amino acid, proline, whose arrangement is in the form of a repeated motif "Pro-X". It also has

another arrangement of amino acids into a "DALK" motif. Other characteristics include defined hydrophobic and hydrophilic domains (Figure 4) and a membrane spanning cleavable signal peptide. the pSAC51 deduced amino acid sequence has an unknown function but has significant homology to other characterised protein whose functions are also unknown (Figure 5). These proteins are from different plant species and from different plant organs; carrot embryos (Aleith and Richter, *Planta* 183 17-24 (1990)) and young maize roots (John *et al*, *Plant Mol. Biol.* 20 821-831 (1992)). The pSAC51 protein also has significant homology (>40%) with a protein of unknown function from immature tomato fruit (Salts *et al*, *Plant Mol. Biol.* 17 149-150 (1991)). this protein is also rich in proline (but has a different arrangement) and has a pronounced hydrophobic domain as well as the "DALK" motif but the cDNA and protein is much larger. Sequence alignment analysis shows the homology to be with proline residues and with the hydrophobic domain particularly with the cysteine residues. There are also other proteins that have significant homology with pSAC51 and were isolated from soybean seeds (Odani *et al*, *Eur. J. Biochem.* 162 485-491 (1987), Estanyol *et al*, *Plant Cell* 4 413-423 (1992)). Again they are proline-rich and have hydrophobic domains. This may give some insight into the role of the protein encoded by pSAC51 in that it may be connected with the developing seed. Given that the pSAC51 protein is likely to be transported and that the seed attachments to the pod occur in the region of the dehiscence zone then it may have a role in seed development, although no pSAC51 mRNA was detected by Northern analysis.

The processes of abscission and dehiscence involve the breakdown of cell walls. The cell walls of a plant are composed of cellulose, hemicellulose, pectic compounds, proteins, suberin, lignin and water (Cassab and Verner, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39 321-353 (1988)), but can be grouped into three main types: hydroxyproline-rich glycoproteins (HRGPs) (Chen and Varner, *Proc. Natl. Acad. Sci.* 82 4399-4403 (1985),

Zheng-Hua and Varner, *Plant Cell* 3 23-37 (1991)); glycine-rich proteins (GRPs) (Condit and Meagher, *Nature* 323 178-181 (1986)); and lastly proline- (or hydroxyproline)-rich proteins (PRPs) (Hong *et al*, *Plant Cell* 1 937-943 (1989), Wyatt *et al*, *Plant Cell* 4 99-110 (1992)). all these

5 proteins are characterised by basic repeat motifs that are different for each type: Ser-(Hyp)₄ for HRGPs; (Gly-X)_n for GRPs; and Pro-Pro-Val-X-Y for PRPs. The proline-rich protein encoded by pSAC51 shows no significant identity to any of the aforementioned groups described so far (Cassab and Varner, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39 321-353

10 (1988)). The proline-rich proteins that do show homology to pSAC51 have all been characterised since that review was written and, given the different proline repeat motif "Pro-X", they may be a new sub-group of proline-rich proteins. Despite these differences pSAC51, and the other

15 proteins it shares homology with, do have elements that link them to the other groups of proline-rich proteins. They often include signal sequences, glycosylation sites and are expressed in specific organs and tissues. The pSAC51 deduced amino acid sequence has one glycosylation site as does the maize protein (John *et al*, *Plant Mol. Biol.* 20 821-831 (1992)) but the tomato (Salts *et al*, *Plant Mol. Biol.* 17 149-

20 150 (1991)) and carrot (Aleith and Richter, *Planta* 183 17-24 (1990)) proteins do not. Many proline-rich proteins that have been characterised so far have been isolated from tissue capable of growth and cell expansion and they may have a role in cell wall formation and structure (Cassab and Varner, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39 321-353

25 (1988)). Others have been found to be produced in response to wounding and stress signals (Zhous *et al*, *Plant Mol. Biol.* 20 5-17 (1992)). The pSAC51 mRNA is present and increases when the pod has stopped growing and so the protein translated from this may be involved in some other process, for example, pod dehiscence. More and more

30 proline rich proteins are being isolated and characterised (Cheung *et al*, *The Plant J.* 3 151-160 (1993), Estanyol *et al*, *Plant Cell* 4 413-423 (1992), Roberts *et al*, *The Plant J.* 3 111-120 (1993), Wright *et al*, *The*

Plant J. 3 41-49 (1993)) and this may help in assigning precise functions for these unknown proline-rich proteins.

EXAMPLE 2 - Cloning of pSAC25/pSAC40

5 Following the general procedure of Example 1, other cDNAs were cloned. One was designated pSAC25, which has a different pattern of gene expression from that of pSAC51. pSAC40 is one of several cDNAs with sequences that match pSAC25: the insert is slightly larger than pSAC25.

10 Pods were harvested at 20, 30, 35, 40, 45, 50 and 60 DAA, and dehiscence zone and flanking non-zone tissue isolated, as described in Example 1. Northern analysis of extracted RNA reveals that expression was taking place in zone tissue from 30 DAA, with only slight expression in non-zone tissue as late as 50 DAA (Figure 7). The transcript could not
15 be detected in leaves, seeds or roots.

The cDNA sequence of pSAC40 was sequenced and the result, with the deduced amino acid sequence, is shown in Figure 8. The hydropathy plot (Figure 9) of the deduced amino acid sequence shows distinct
20 hydrophobic and hydrophilic regions.

Genomic Southern analysis of pSAC25

The insert of pSAC25 was used as a probe for hybridisation to Southern blots of *B. napus* genomic DNA digested with *EcoRI*, *HindIII* and *BamHI*.
25 The probe hybridised to several fragments ranging in size from 7 or 8 kb to 1 kb.

EXAMPLE 3 - Cloning of gSAC51 and gSAC51

a) Isolation and characterisation of the SAC25 gene.

30 A 10 kb *A. thaliana* genomic clone [gSAC25] was isolated that hybridised to the pSAC25 cDNA (Figure 11A). A 3.5 kb *EcoRI/HindIII* fragment of this DNA that hybridised to the pSAC25 cDNA was subcloned into

EcoRI/HindIII cut pBluescript KS⁺, forming pDH30. Nucleotide sequencing shows that this fragment contains an ORF that is highly homologous to the pSAC25 ORF. This sequence also determines the position of the SAC25 promoter region relative to the SAC25 ORF, as it is that region upstream of (ie 5' to) the ORF.

b) Isolation and characterisation of the SAC51 gene.

A 10 kb *A. thaliana* genomic clone [gSAC51] was isolated that hybridised to the pSAC51 cDNA (Figure 11B). A 4.0 kb *EcoRI* fragment of this DNA that hybridised to the pSAC51 cDNA was subcloned into *EcoRI* cut pBluescript KS⁺, forming pDH31. Nucleotide sequencing shows that this fragment contains an ORF that is highly homologous to the pSAC51 ORF. This sequence also determines the position of the SAC51 promoter region relative to the SAC51 ORF, as it is that region upstream of (ie 5' to) the ORF.

EXAMPLE 4 - Use of SAC25 and SAC51 promoters

To demonstrate that the putative promoter regions of SAC25 and SAC51 are capable of driving the expression of a foreign gene in *A. thaliana*, *B. napus* and *N. tabacum*, transcriptional fusions of the promoters can be made to the *E. coli* gene encoding β -glucuronidase (GUS). Fragments of the clones [gSAC25] or [gSAC51] containing the putative promoter region are subcloned into pBI101 (Jefferson *et al*, *EMBO J.* 6 3901 (1987)). The GUS constructs are then transformed into *A. thaliana*, *B. napus* or *N. tabacum*, using standard transformation techniques. Analysis of the transformed plants demonstrates that GUS activity is localised to the pod shatter zone. The temporal regulation of GUS activity will be identical to the temporal expression observed for the SAC25 and SAC51 genes as described in Examples 1 and 2.

EXAMPLE 5 - The Construction of Expression Cassettes and their use in Producing Sense and Anti-Sense RNA to Pod Shatter Zone-Specific Messages in Transgenic Plants

5 Either pod shatter zone-specific or constitutive promoters can be used to drive expression of sense or anti-sense RNA corresponding to shatter zone-specific transcripts in transgenic plants, thus potentially creating pod mutations and shatter-resistance (indehiscence). The same pod shatter zone-specific promoters can be used to drive the pod shatter zone expression of genes encoding proteins or enzymes detrimental to shatter-
10 zone function thereby creating shatter-resistance (indehiscence).

a) Construction of an Intermediate Vector to Express Sense and Anti-Sense RNA Utilising the Double 35S Promoter

15 A 650 bp *Hind*III, *Eco*RI SAC25 fragment from the SAC25 cDNA was cloned into *Hind*III, *Eco*RI-cut pJIT60 forming p35S-antiSAC25 (Figure 12). pJIT 60 is identical to pJIT30 (Guerineau *et al*, *Plant Mol. Biol.* 15 127-136 (1990)) except that the CaMV 35S promoter is replaced by a double CaMV 35S promoter. In p35S-antiSAC25 a portion of the SAC25 cDNA is cloned in an antisense orientation between a double 35S
20 promoter and a CaMV polyadenylation signal. This chimeric gene was then cloned into pBin19 (Bevan *et al*, *Nucl. Acids Res.* 22 8711-8721 (1984)) as a *S*sn, *X*hoI fragment forming p35S-antiSAC25. *B. napus* plants transformed with the 35S-antiSAC25 chimeric gene are resistant to pod-shatter (indehiscent).

25

Other chimeric genes that can be constructed to produce shatter-resistance include, by way of non-limiting example:

30 i) Double CaMV 35S promoter linked to the coding region of the SAC25 cDNA or gene such that sense SAC25 RNA is produced;

ii) Double CaMV 35S promoter linked to the coding region of the SAC51 cDNA or gene such that sense or anti-sense SAC51 RNA is produced;

5 iii) SAC25 promoter linked to the coding region of the SAC25 cDNA or gene, such that sense or anti-sense SAC25 RNA is produced;

iv) SAC51 promoter linked to the coding region of SAC25 cDNA or gene, such that sense or anti-sense SAC25 RNA is produced;

10 v) SAC25 promoter linked to the coding region of the SAC51 cDNA or gene, such that sense or anti-sense SAC51 RNA is produced; and

vi) SAC51 promoter linked to the coding region of the SAC51 cDNA or gene, such that sense or anti-sense SAC51 RNA is produced.

15

These plasmids could also be transformed into other members of the *Brassicaceae* causing shatter-resistance in the transgenic plants.

20 The utility of the SAC25 and SAC51 promoters could also be harnessed by expressing gene fusions to barnase, or other genes that disrupt cellular development or otherwise interfere in the function of the shatter zone in pod shatter, in transgenic plants. Use of the barnase gene to cause cell ablation has been described in EP-A-0344029 (Plant Genetic Systems NV) and WO-A-9211379 (Nickerson International Seed Company Limited),

25 particularly at pages 28 and 29 of the latter document.

Transcriptional or translational fusion of the SAC25 or SAC51 promoter fragments and the transfer of these genes into *B. napus* or *N. tabacum* plants results in ablation of the pod shatter zone causing shatter-resistance.

30

CLAIMS

1. A recombinant or isolated nucleic acid sequence which:
 - (a) encodes an enzyme or other protein involved in plant abscission or dehiscence;
 - (b) contains a promoter or other regulatory sequence which naturally controls expression of a gene involved in plant abscission or dehiscence;
 - (c) when introduced into a plant, prevents or otherwise interferes with normal plant abscission or dehiscence; or
 - (d) hybridises under stringent conditions to nucleic acid satisfying criterion (a), (b) or (c) or would do so but for the degeneracy of the genetic code.
2. Nucleic acid as claimed in claim 1, wherein the dehiscence is dehiscence of *Brassica napus*.
3. Nucleic acid as claimed in claim 1(a), which is either the nucleic acid sequence of Figure 3 or 8, or a degenerate sequence thereof, or a nucleic acid sequence which is substantially homologous to a nucleic acid sequence encoding the amino acid sequence shown in Figure 3 or 8.
4. Nucleic acid as claimed in claim 1(a) which encodes a protein specifically or at least preferentially expressed in the dehiscence zone of pericarp tissue of *Brassica napus*.
5. Nucleic acid as claimed in claim 1(b), wherein the promoter is operatively coupled to a DNA sequence.
6. Nucleic acid as claimed in claim 5, wherein the promoter is operatively coupled to DNA coding a lytic enzyme.

7. Nucleic acid as claimed in claim 6, wherein the lytic enzyme is a ribonuclease, protease or glucanase.
8. Nucleic acid as claimed in claim 5, wherein the promoter is operatively linked to DNA encoding antisense RNA or a ribozyme.
9. Nucleic acid as claimed in claim 1(c) which interferes with the proper expression of one or more genes involved in abscission or dehiscence.
10. Nucleic acid as claimed in claim 9 which comprises antisense DNA or ribozyme-encoding DNA specific for an abscission- or dehiscence-specific gene operatively coupled to a constitutive or other promoter.
11. Nucleic acid as claimed in claim 10, wherein the promoter is the CaMV 35S, rubisco or plastocyanin promoter.
12. Nucleic acid as claimed in any one of claims 1 to 11 comprising a 3'-transcription regulation signal.
13. Nucleic acid as claimed in any one of claims 1 to 12, which is in the form of a vector.
14. A host cell transfected or transformed with nucleic acid as claimed in any one of claims 1 to 13.
15. A process for preparing nucleic acid as claimed in any one of claims 1 to 13, the process comprising coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides.
16. A plant cell including nucleic acid as claimed in any one of claims 1 to 13.

17. A whole plant, or part of a plant, comprising cells as claimed in claim 16.
18. A protein which is preferentially or exclusively expressed in a dehiscence zone or abscission layer off a plant.
19. A protein as claimed in claim 18, which has the amino acid sequence shown in Figure 3 or 8 or a sequence substantially homologous thereto.

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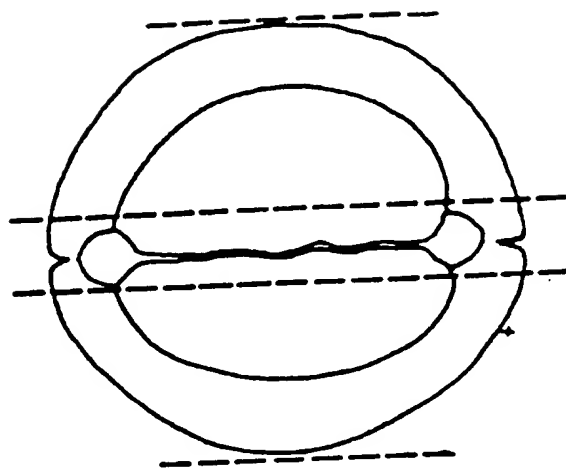


FIG. 1

NON-ZONE (VALVE)

ZONE

FIG. 4

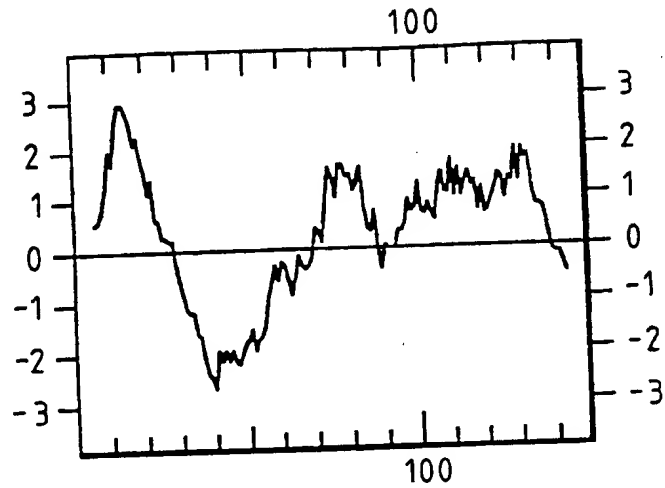
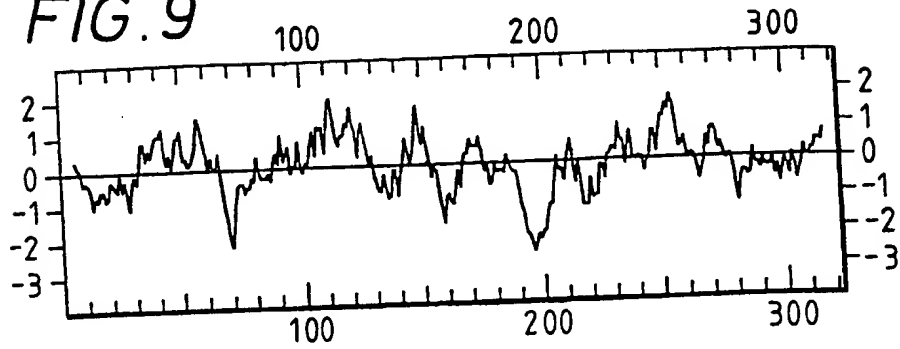


FIG. 9



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FIG. 2

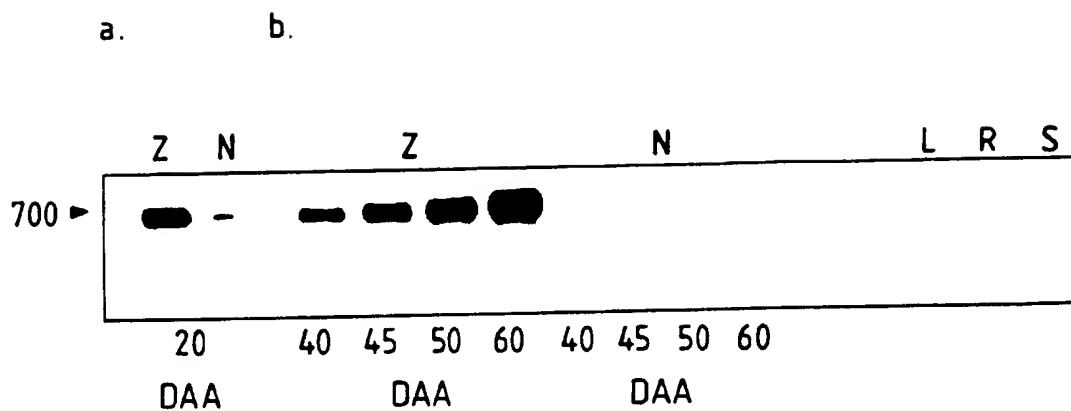
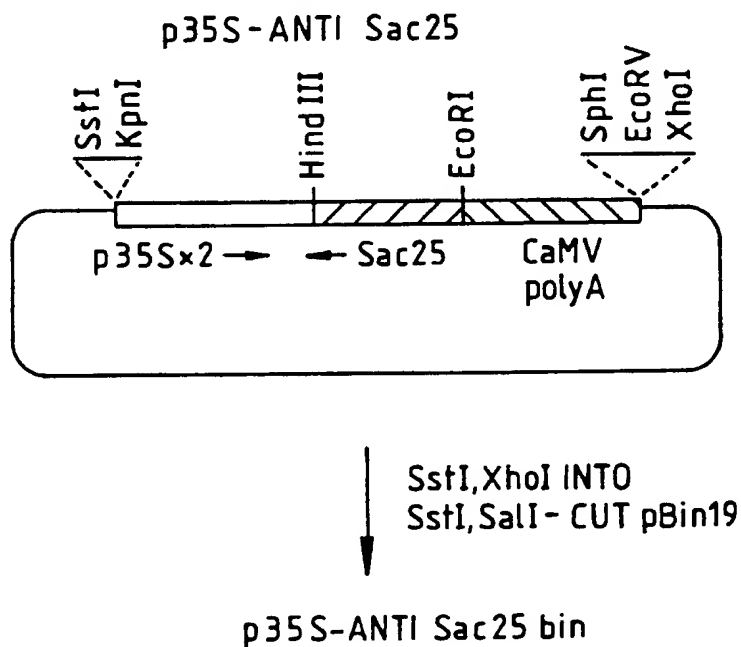


FIG. 12



KEY TO SEQUENCES

- DOUBLE 35S CaMV PROMOTER
- ▨ Sac25 cDNA
- ▧ CaMV POLYADENYLATION SIGNAL

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*M A S R T K S F L A I
 AGAGGAATTAAACA ATG GCT TCA AGA ACG AAA AGC TTT TTA GCC ATT
 F L I L N I L F C T T I S A Y
 TTC TTG ATT CTG AAC ATC CTT TTC TGC ACA ACA ATC TCT GCC TAC
 G N C G C P S P K P K P D P S
 GGT AAC TGC GGT TGC CCT TCT CCC AAG CCA AAA CCT GAC CCC TCC
 H K P K P N P K P K P T P T P
 CAT AAG CCA AAA CCT AAC CCT AAA CCC AAA CCA ACC CCA ACT CCA
 T P S P V T A K C P R D A L K
 ACC CCT AGC CCT GTC ACA GCC AAA TGC CCT AGA GAC GCT CTT AAA
 L G V C A N V L S G L L N I T
 CTA GGA GTC TGC GCC AAC GTG CTC AGC GGT CTA CTC AAC ATC ACC
 L G K P P V K P C C T L I K G
 CTT GGG AAG CCA CCT GTG AAG CCA TGT TGC ACC CTC ATC AAA GGA
 L A D L E A A C L C T A L K
 CTT GCT GAT CTT GAA GCC GCG GCT TGT TGC ACC GCG CTT AAG
 A N I L G I N L N I P I S L S
 GCT AAC ATC CTT GGG ATC AAC CTG AAC ATC CCT ATC TCA CTC AGT
 L L L N V C S K K V P P G F Q
 CTG CTT CTC AAT GTT TGT AGC AAA AAG GTT CCC CCT GGT TTC CAA
 C *Z
 TGC TAA TCAAGATTATAATTATACAACCACCACCTGGATGTCAACATATATACTCTT
 GTTGGATAGACAAGATAATATATGTAAATATAGATTCTGTAGTATTTCTGTGTTTAT
 GTATGAATTGTATGTGTGTATGTGATTTCTACAACTCTAAACTTCACATTTGTTTT
 ATTTTGTCTCTTAATTATATATACAGTCACAGGGGTGTGTGTACTGTGTGTTTT

 AAATTAATAATAATATGTTTAATACTGAATAAAAAAAAAAAAAAAAAAAAAA
 AAAAAAAAAA

FIG. 3

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FIG. 5

1	1	MASRTKSFLA	IFLILNIFC	TTISAYGNCG	CPSPKPKPDP	SHKPKPNPKP	50
PSAC51		MGSKNSASVA	LFFTLNILFF	ALVSSTEKC.PDP	.YKPKPKPTP	
DC2.15		MAPK....VA	LFLALSLLFA	ATAHGCE...P	NCSGPPVVPTP	
pZRP3							
	51	KPTPTPTPSP	VTAKCPRDAL	KLGVCANVLS	GLLNITLTKP	PVKPCCTLIK	100
PSAC51		KPTPTPYPSA	..GKCPRDAL	KLGVCAADVLN	LVHNVVIGSP	PTLPCCSLLE	
DC2.15		PVVPTP.SSH	SHGRCPIDAL	KLKVCACVLG	L...VKVGLP	QYEQCCPLLE	
pZRP3							
	101	GLADLEAAAC	LCTALKANIL	GINLNIPISL	SLLLNVCSSK	VPPGFQC..	149
PSAC51		GLVNLEAAVC	LCTAIKANIL	GKNLNLPIAL	SLVLNCGKQ	VPNGFECT.	
DC2.15		GLVDLDAALC	LCTAIKANVL	GIHLNVPLSL	NFILNCGRI	CPEDFTCPN	
pZRP3							

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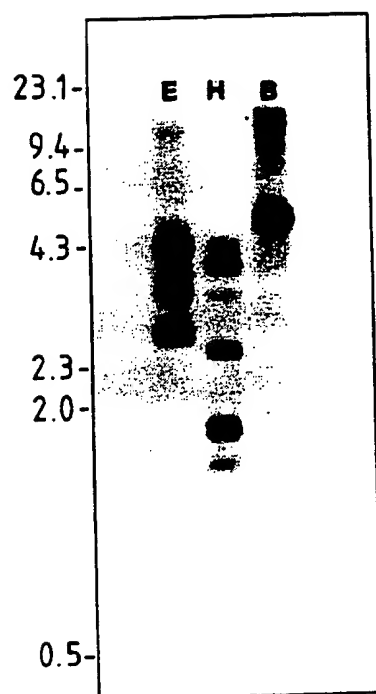


FIG. 6

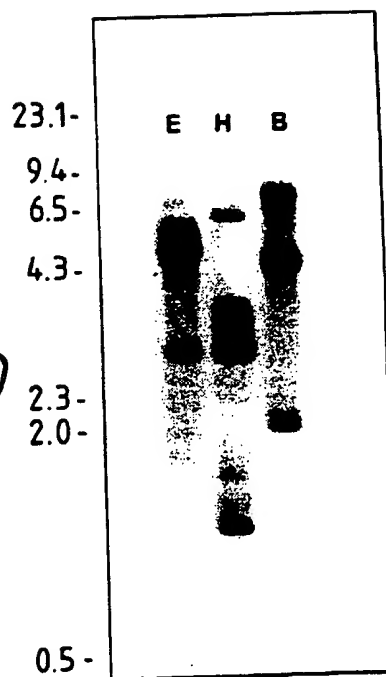
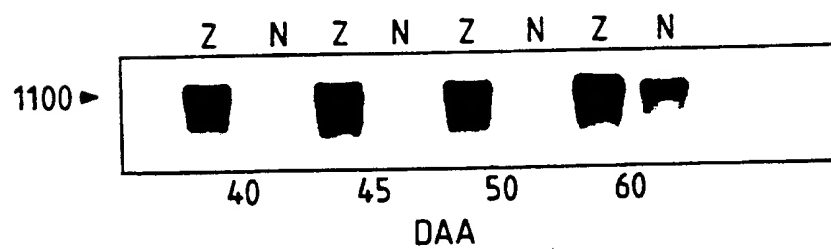
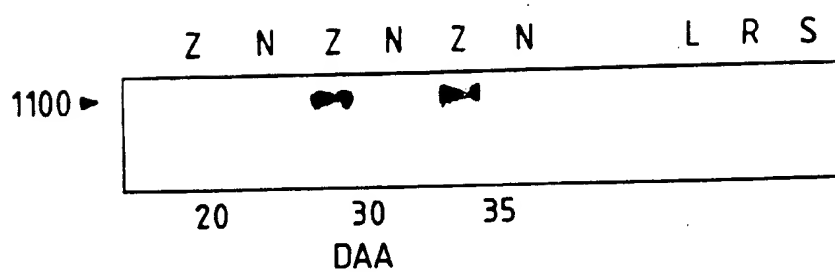


FIG. 10

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FIG. 7



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FIG. 8

AAATTGAGAGT	TTTATCTTCTCTCCTGTACATTA	TTCTTCTTCAATCTGC	52
	*M	E I Y G	5
AACATTTTAA	TAGGGTTTCTTAGTTTGGAGAGACATC	ATG GAG ATA TAT GGA	106
M V T G K A G K S G Y G S A S			20
ATG GTT ACA GGA AAA GCA GGA AAG AGT GGA TAC GGA TCA GCA TCA			151
T A E D V T H S I D A K H L T			35
ACA GCT GAA GAT GTC ACT CAC TCC ATT GAT GCC AAA CAT CTC ACT			196
A I I T G G T S G I G L E A A			50
GCC ATC ATC ACA GGT GGA ACA AGT GGG ATT GGA TTA GAA GCA GCA			241
R V L G M R G A H V I I A S R			65
AGA GTG TTG GGA ATG AGA GGA GCT CAT GTC ATT ATC GCG TCA AGA			286
N T K A A N D S K E M I L Q M			80
AAC ACA AAA GCA GCT AAC GAT TCT AAA GAG ATG ATT CTT CAG ATG			331
Y P N A R I D C L Q L D CTC TCT TCT			95
TAC CCT AAT GCA CGC ATC GAC TGT CTT CAG CTT F L A L N			376
I K S V R S F I H Q CAG TTT CTT GCC CTT AAT			110
ATC AAA TCC GTC AGA TCC TTC ATC CAT CAG TTT CTT GCC CTT AAT			421
V P L N I L I N A G G T V M F C			125
GTC CCT CTC AAC ATA CTC ATA AAC AAT GCA GGT GTT ATG TTC TGT			466
P F Q L S E D G I E S Q F A T			140
CCT TTC CAG CTC AGT GAA GAT GGG ATT GAA TCA CAA TTC GCA ACA			511
N H I G H F L L T N L L D K			155
AAC CAC ATT GGT CAT TTT CTG TTG ACG AAT CTT CTT CTG GAC AAA			556
M K S S A R E S G I E G R I V			170
ATG AAG AGT AGT GCA CGA GAA AGT GGG ATC GAA GGA AGG ATC GTG			601
N L S S I A H T Y T Y T E G I			185

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AAT	CTG	TCA	TCT	ATC	GCT	CAT	ACT	TAT	ACT	TAC	ACC	GAA	GGC	ATA	646										
M	F	D	Y	I	N	D	P	D	R	Y	S	E	K	K	200										
ATG	TTC	GAT	TAC	ATC	AAC	GAC	CCA	GAT	CGA	TAT	TCT	GAG	AAA	AAA	691										
A	Y	G	Q	S	K	L	A	N	L	L	H	S	N	A	215										
GCT	TAT	GGA	CAG	TCA	AAA	CTG	GCA	AAC	TTA	TTG	CAC	TCC	AAT	GCA	736										
L	S	R	K	L	Q	E	E	G	V	N	I	T	I	N	230										
CTC	TCT	CGT	AAA	CTA	CAG	GAG	GAA	GGT	GTG	AAC	ATC	ACA	ATA	AAC	781										
S	V	H	P	G	L	I	T	T	N	L	F	R	H	S	245										
TCG	GTA	CAC	CCT	GGA	CTT	ATA	ACC	ACT	AAT	CTC	TTT	CGT	CAC	TCC	826										
G	L	G	M	A	V	L	K	A	M	S	F	F	L	W	260										
GGT	TTA	GGA	ATG	GCG	GTC	CTC	AAG	GCT	ATG	AGC	TTC	TTT	TTA	TGG	871										
K	N	I	P	Q	G	A	A	T	T	C	Y	V	A	L	275										
AAA	AAC	ATA	CCA	CAG	GGA	GCA	GCA	ACG	ACA	TGC	TAC	GTG	GCA	CTT	916										
H	P	D	L	K	D	V	T	G	K	Y	F	A	D	C	290										
CAT	CCT	GAT	TTA	AAA	GAC	GTC	ACC	GGG	AAG	TAC	TTC	GCG	GAC	TGT	961										
N	V	T	T	P	S	N	F	A	T	D	T	T	L	A	305										
AAC	GTC	ACC	ACT	CCA	AGT	AAC	TTT	GCC	ACC	GAC	ACT	ACC	CTC	GCC	1006										
D	K	L	W	D	F	S	I	K	L	V	E	S	L	P	320										
GAT	AAA	CTT	TGG	GAT	TTC	AGT	ATA	AAA	CTC	GTC	GAG	TCT	CTT	CCC	1051										
*																									
TAA	CTATATATCTAAACGAATT	TTGTACTCCATAATGTTT	TACATTAAATTTT	TATCAGC	1109																				
ACATATTTGTTTATGGAACTAATAT	TATATATCAGAAACACCAATTG	AAAAAATAAAAAATG	1168																						
AAATGTAAC	TAATAAAAAA	AAAAA	1197																						

FIG. 8 (contd.)

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FIG. 11A

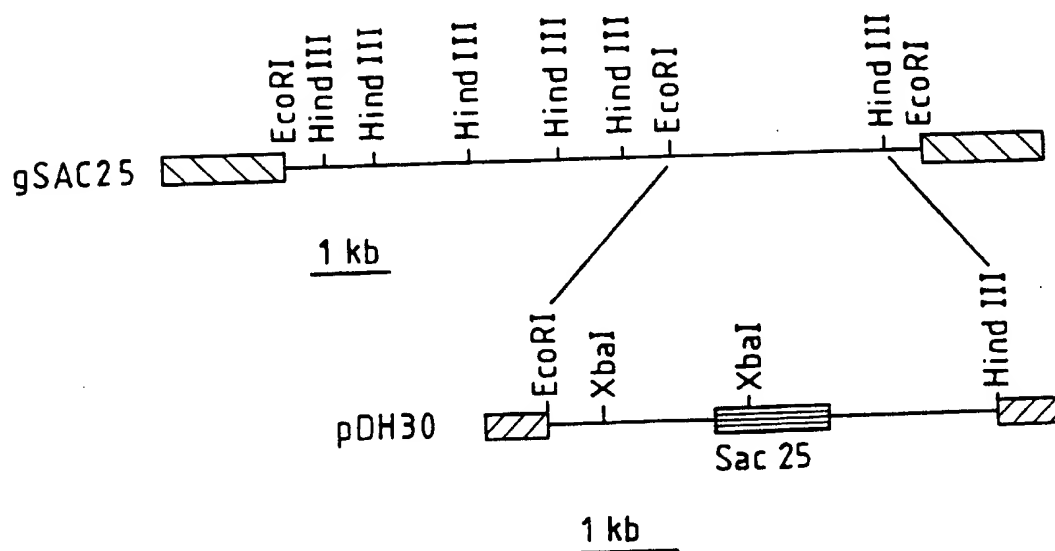
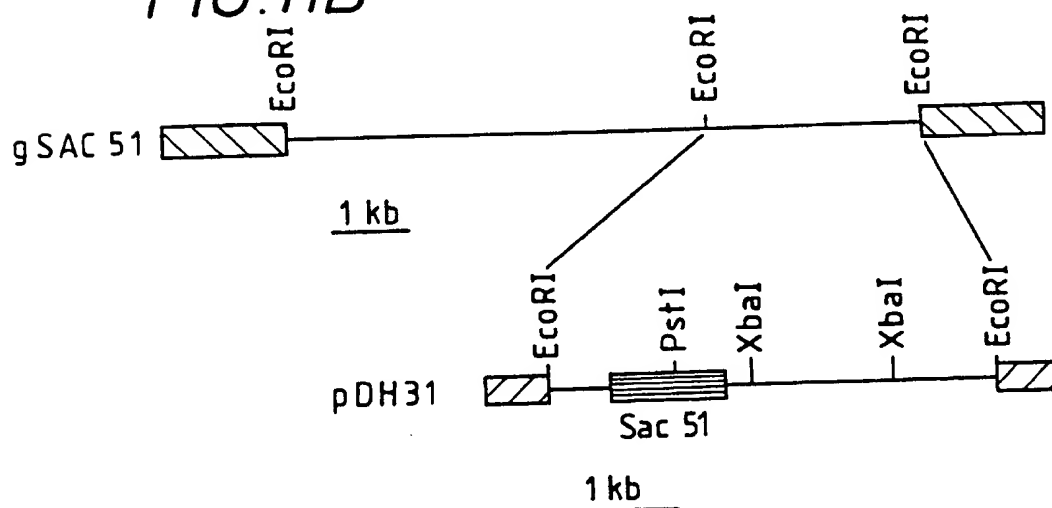





FIG. 11B



Key to DNA sequences

-  Lambda Dash
-  pTZ vector
-  Sac25 or Sac51 coding region
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